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TITLE OF THE INVENTION (280 Characters max)

MUTATIONS IN NOD2 ARE ASSOCIATED WITH FIBROSTENOSING DISEASE IN PATIENTS WITH CROHN'S DISEASE

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ENCLOSED APPLICATION PARTS (*check all that apply*)

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Respectfully submitted,

SIGNATURE Andrea K. Gashler Date August 30, 2002

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Additional inventors are being named on separately numbered sheets attached hereto.

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PROVISIONAL

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on

MUTATIONS IN NOD2 ARE ASSOCIATED WITH FIBROSTENOSING DISEASE IN PATIENTS WITH CROHN'S DISEASE

by

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MUTATIONS IN NOD2 ARE ASSOCIATED WITH FIBROSTENOSING DISEASE IN PATIENTS WITH CROHN'S DISEASE

Running title: NOD2 is associated with fibrostenosing Crohn's disease

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Abbreviations: SNP= single nucleotide polymorphism; pANCA= perinuclear anti-neutrophil cytoplasmic antibody; ASCA= anti-saccharomyces cerevisiae antibody; TLR= toll-like receptor; PAMP= pathogen-associated molecular pattern; LPS= lipopolysaccharide

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Abstract

Background and Aims: The clinical manifestations of Crohn's disease (CD) are diverse, ranging from fibrostenosing small bowel disease to colon-predominant inflammation. These distinctions may represent genetic, immunologic and microbial heterogeneity. NOD2 gene mutations in Crohn's disease have been described recently and may alter innate immune responses. We hypothesized that NOD2 mutations may be associated with distinct phenotypic expressions of Crohn's disease. Methods: Two cohorts of consecutively-identified patients referred to an IBD center (n=142 collected between 1993-96; n=59 collected between 1999-2001) were genotyped for three allelic variants of NOD2, R675W, G881R, and 3020insC, and phenotyped for disease behavior, disease location and serum immune markers. Results: Univariate analysis revealed that CD-associated NOD2 variants are significantly associated with fibrostenosing disease in each cohort (p=0.049 and p=0.002, respectively). When both cohorts were analyzed together, the association between NOD2 variants and fibrostenosing disease was more

significant (p= 0.001). These relationships were observed in both Jews and non-Jews. Forty-six percent of patients with fibrostenosing disease carry at least one of these alleles, compared to only 23.5% of patients without fibrostenosing disease (OR 2.8, 95% CI 1.6- 5.2). Multivariate and conditioning analyses revealed a primary association between NOD2 allelic variants and fibrostenosing disease but not with small bowel disease. Conclusion: In this description of a genotype-phenotype correlation in CD patients and NOD2 variants, data suggest that variation in this gene contributes to the occurrence of fibrostenotic CD of the small bowel.

Key words: Crohn's disease, NOD2, fibrostenosing disease, genetic heterogeneity, phenotypic heterogeneity.

Introduction

Crohn's disease is a phenotypically heterogeneous disorder with diverse clinical manifestations. It may be characterized at multiple levels. Clinicians have characterized Crohn's disease based on the location of the disease, i.e. small bowel, colon or both, or based on complications of the disease, i.e. stricturing or perforating [1-3]. Crohn's Disease may also be classified by expression of serum immune markers such as ASCA and pANCA [2-4], or by response to therapy, e.g. steroid-dependent or infiximab responsive [5-7]. At the root of these disparate clinical manifestations lies an interplay of genetic, immunologic, and possibly microbial factors that culminates in distinct phenotypic expressions of the disease.

Animal models have provided novel insights into the pathogenesis of human inflammatory bowel disease and identified candidate immunologic pathways that result in intestinal inflammation. These studies have also highlighted the need for commensal bacteria to unleash the host susceptibility [8-12]. Among the abnormalities identified in patients with Crohn's disease is the finding of immunologic reactivity towards the individual's microbial flora whereas healthy controls are immunologically tolerant to the indigenous flora [13]. Thus, inflammatory bowel disease may result from an abnormal mucosal immune response to commensal bacteria or unidentified pathogens.

Sensing of bacterial products by the innate immune system is mediated by a family of receptors, toll-like receptors (TLRs), which activate the transcription factor NF-□B in response to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS). TLR4 is required for recognition of LPS [14-17]. We have recently described that intestinal epithelial cells express low levels of TLR4 and are LPS unresponsive [18] suggesting that one mechanism by which the mucosal immune system is protected against chronic inflammation is down-regulation of TLR signaling molecules. Nuñez and colleagues have recently identified the NOD2 gene [19]. Its protein product is a member of the Apaf-1/Nod1 family of caspase-recruitment domain (CARD)-containing proteins and is homologous to disease-resistance genes found in plants [20]. NOD2 is expressed by monocytes and activates NF-□B in response to LPS. Several recent studies have identified a frameshift mutation in NOD2 (3020insC), in patients with

Crohn's disease that results in a 33 amino acid truncation of the protein [21-23]. The frameshift mutation and an additional polymorphism of NOD2 associated with Crohn's disease are located in the leucine-rich region of the gene suggesting that these rare alleles may affect the ability of NOD2 to interact with other intracellular proteins. Expression of this truncated mutant of NOD2 in 293T cells dampens NF-□B activation in response to LPS [22]. Since the original identification of three allelic variants of NOD2 associated with Crohn's disease, mutational analysis in 453 patients with Crohn's disease has identified an additional 27 rare mutations accounting for 19% of all NOD2 mutations [24]. Greater than 90% of all the Crohn's-associated NOD2 mutations are located in the distal third of the gene suggesting that these may also affect the function of NOD2 with respect to bacterial recognition and signaling. Patients with a related syndrome, Blau's syndrome, characterized by granulomatous inflammation have distinct mutations in NOD2 within the nucleotide-binding domain of the gene [25]. These data support the notion that innate immunity is altered in patients with Crohn's disease resulting in abnormal immune responses to commensal or pathogenic bacteria.

We have previously described that the serum immune markers pANCA and ASCA are associated with distinct clinical phenotypes of Crohn's disease [2, 3]. Studies have demonstrated that these antibodies recognize bacterial or yeast PAMPs [26-29]. Because NOD2 regulates responses to bacterial products in monocytes, we hypothesized that expression of NOD2 mutants may be associated with specific clinical phenotypes of Crohn's

disease. The results of our studies reported herein demonstrate that mutations in NOD2 occur more frequently in patients with stricturing, i.e. fibrostenotic, Crohn's disease of the small bowel. These results have important implications for the identification of host-microbial interactions resulting in stricturing complications of Crohn's disease.

Materials and Methods

Human subjects: Two cohorts of patients were examined in the current study. Both cohorts were consecutively identified Crohn's disease patients from an inflammatory bowel disease referral center (Cedars-Sinai Medical Center Inflammatory Bowel Disease Center). The first cohort (Cohort 1) (n=142) was ascertained between 1993-96 and has been previously described [2]. The second cohort (Cohort 2) (n=59) was collected between 1999-2001. Thus, the study population consisted of 201 consecutively ascertained patients evaluated by the CSMC IBD Center, with an established diagnosis of CD. A cohort of 175 patients with ulcerative colitis were used as an inflammatory disease control group. This study was reviewed and approved for human subject participation by the Cedars-Sinai Institutional Review Board and permitted the collection of clinical, serologic and genetic data from patients consenting to the study. Diagnosis of CD was defined by the presence of a combination of established features from *at least two* of the following categories: 1) clinical - perforating or fistulizing disease, obstructive symptoms secondary to small bowel stenosis or stricture; 2) endoscopic - deep

linear or serpiginous ulcerations, discrete ulcers in normal-appearing mucosa, cobblestoning, discontinuous or asymmetric inflammation; 3) radiographic - segmental disease (skip lesions), small bowel or colon strictures, stenosis, or fistula, *and/or;* 4) histopathologic - submucosal or transmural inflammation, multiple granulomas, marked focal cryptitis or focal chronic inflammatory infiltration within and between biopsies, or skip lesions including rectal sparing in the absence of local therapy.

Phenotypic analyses: Patients with Crohn's disease were characterized as having fibrostenosing disease, internal-perforating disease, perianal fistulizing disease or ulcerative colitis (UC)-like disease based on previously described criteria [1-3, 31]. Briefly, patients were considered to have fibrostenosing disease if they had documented persistent intestinal obstruction and/or required an intestinal resection for an intestinal obstruction. Internal perforating disease was recorded if patients had current or previous evidence of entero-enteric or entero-vesicular fistulae, intraabdominal abscesses, or small bowel perforation. Perianal perforating disease was recorded if patients had current or previous evidence of either perianal fistulae or abscesses or rectovaginal fistula. Finally, UC-like disease was recorded if patients had current or previous evidence of left-sided colonic involvement, symptoms of bleeding and/or urgency, and crypt abscesses on colonic biopsies as previously described [3]. Disease location was classified as small bowel, colon, or both based on endoscopic, radiologic and/or pathologic studies. A panel

of inflammatory bowel disease physicians (MTA, EAV, LYK, KP, SRT) masked to the results of serologic or genetic testing reached a consensus on phenotype based on the clinical data.

Genotyping: Using the software PrimerExpress 1.5™(PE Biosystems, Foster City, CA), the sequence information found in dbSNP (www.ncbi.nlm.nih.gov) for the NOD2 R675W, G881R, and 3020insC mutations (also referred to as SNP, 8,12, 13) [21] as well as SNP 5, the background allele for these mutations, was used to design genotyping assays employing 5'-exonuclease technology,[32] also known as the TaqMan MGB™ assay (PE Biosystems, Foster City, CA). The MGB™ design adds a "minor groove binder" to the 3'-end of the TaqMan™ probes that increases the binding temperature of the probe and thus enables shorter probes to be used than in conventional TaqMan™ assays [33]. This has the effect of increasing the discrimination between the alleles in the assay.[34]. Assays were performed following the manufacturer's recommendations (PE Biosystems bulletin 4317594) in an ABI 7900 instrument. Genotyping was done blinded to clinical status of the subjects.

	PCR		TaqMan Probes
SNP	Forward primer	Reverse primer	
5	5'-GGG TGG CTG GGC TCT TCT-3'	5'-CTC GCT TCC TCA GTA CCT ATG ATG-3'	5'FAM-CAT GGC TGG ACC C-MGBNFQ 5'TET-CAT GGC TGG ATC C-MGBNFQ
8	5'-GGC GGG ATG GAG TGG AA-3'	5'-CTG GCT GAG TGC CAG ACA TCT-3'	5'FAM-TGC TCC GGC GCC A-MGBNFQ 5'TET-CTG CTC

			TGG CGC CA-MGBNFQ
12	5'-CCA CCT CAA GCT CTG GTG ATC-3'	5'-GTT GAC TCT TTT GGC CTT TTC AG-3'	5'FAM-CTG TGT TGC CCC AGA A-MGBNFQ 5'TET-CTC TGT TGC GCC AGA-MGBNFQ
13	5'-CCT TAC CAG ACT TCC AGG ATG GT-3'	5'-TGT CCA ATA ACT GCA TCA CCT ACC T-3'	5'FAM-CCT TTC AAG GGG CCT-MGBNFQ 5-TET-CTT TCA AGG GCC TGC-MGBNFQ

Serologic analyses: Serum ANCA expression and ANCA subtype characterization were performed by fixed neutrophil enzyme-linked immunosorbent assay (ELISA) as previously described [35]. Briefly, human peripheral blood neutrophils fixed with methanol were reacted with control and coded sera at a 1:100 dilution. Anti-human immunoglobulin G (gamma-chain specific) antibody (Jackson Immunoresearch Labs, Inc., West Grove, PA) conjugated to alkaline phosphatase was added to label neutrophil-bound antibody and a colorimetric reaction performed. Levels were determined relative to a standard consisting of pooled sera obtained from well-characterized $pANCA^+$ UC patients. Results were expressed as ELISA units (EU/ml). $ANCA^+$ sera were further subtyped via indirect immunofluorescent staining to determine the ANCA neutrophil binding pattern as previously described [35]. Sera exhibiting the characteristic perinuclear highlighting and losing its characteristic staining pattern when treated with DNase were termed "$pANCA^+$" [36]. For the purposes of the current study, patients were considered pANCA positive if they were

both positive for ANCA by ELISA and lost perinuclear immunofluorescence staining with DNAase treatment.

Sera were analyzed for ASCA expression in a blinded fashion using a fixed ELISA assay [2, 37]. Two patients in the second cohort did not undergo ASCA testing. High-binding polystyrene microtiter plates were coated with purified phosphopeptidomannans extracted from yeast *Saccharomyces uvarum*, a subspecies of *S. CEREVISIAE*. Coded patient sera were diluted and added to the wells followed by an enzyme-linked colorimetric reaction. Color development was proportional to concentrations of antibody present in the sera. Levels were determined and results expressed as ELISA units (EU/ml), relative to a standard, which is derived from a pool of patient sera with well-characterized Crohn's disease found to have reactivity to this antigen. Sera exhibiting ASCA IgG reactivity >40 EU/ml or IgA reactivity >20 EU/ml were termed "ASCA positive" ($ASCA^+$). Serologic assays were performed at Cedars-Sinai Medical Center and Prometheus Laboratories (San Diego, CA) using identical methodology.

Statistical Analyses: In order to identify clinical features and immunological traits that are associated with allelic variants of the NOD2 gene, our study was designed to analyze two consecutively ascertained cohorts of patients with Crohn's disease. The first cohort was used to explore the relationship of NOD2 alleles with an array of clinical and serologic variables and generate hypotheses. The second cohort was then used to confirm the specific hypotheses generated from analysis of the

first cohort. To minimize the type 1 error and to maximize the statistical power, we permitted the significance of the associations in the first cohort to be less stringent ($p < 0.1$) and used the second, independent cohort to confirm the associations identified in the first cohort ($p< 0.05$). By avoiding a highly stringent correction for the number of variables examined in the first cohort, this strategy has the advantage of increasing the power to identify specific associations between NOD2 and clinical variables especially since some of these traits are known to be associated with each other (e.g. small bowel involvement and ASCA expression).

Since the three rare alleles are independently associated with Crohn's disease [21], we analyzed each of them individually as well as combined. Statistical analysis was performed using SAS computer software (Version 6.10; SAS Institute, Inc., Cary, NC, 1994) [38]. Quantitative variables are described as medians (range) throughout. Nonparametric statistical tests were used to test differences of quantitative variables between two groups. Chi-square test or Fisher's exact test (when the expected number <5) was used to evaluate associations between carriers and non-carriers of the rare alleles or between genotypes and categorical variables, such as type of IBD, disease location, disease behavior, and antibody positivity. Multivariate analysis was performed using the logistic regression model to test the association between genotypes and phenotypic variables that were significantly associated with NOD2 variants from the univariate analyses. In addition, Mantel Haenszel stratified association test was performed for genotype

and phenotype associations by controlling for potential confounding effect due to ethnic variation [39]. This stratified association test was also used to demonstrate whether the association between NOD2 variants and a phenotype (e.g. fibrostenosing disease) was primary or secondary to other related phenotypes (e.g. small bowel involvement).

Results

Patients with Crohn's disease have an increased frequency of rare allelic variants of NOD2

An association between Crohn's disease and allelic variants of NOD2 has been previously described [21-23]. All three studies identified an association between Crohn's disease and an insertion polymorphism in NOD2, 3020insC or 980fs (SNP 13) but only the Hugot study [21] further identified two missense mutations, R675W (SNP 8) and G881R (SNP 12). We first wished to determine whether our North American Crohn's disease referral patient population expressed similar allelic variants of NOD2 and could serve as a relevant population in which to study differences in phenotypic expressions associated with NOD2 variant alleles. To address this question Cohort 1 (hypothesis-generating) and Cohort 2 (hypothesis-confirming) (see Methods) were genotyped for all three variants-R675W (SNP 8), G881R (SNP 12) and 3020insC (SNP 13). The clinical characteristics of these two Crohn's disease cohorts are shown in Table 1. In general, the first cohort demonstrated a higher percentage of patients with perforating and fibrostenotic complications of

disease. These differences may be due to availability of improved therapy for Crohn's disease in the second cohort or more severe Crohn's disease in the first cohort. A cohort of ulcerative colitis patients was used as an inflammatory disease control group. Each of the three allelic variants of NOD2 were significantly more frequent in patients with Crohn's disease compared with ulcerative colitis (Table 2). As can be seen in Table 2, the frequency of each of the NOD2 variants was extremely similar in each cohort of Crohn's disease patients, supporting their combined use in the final analysis. The overall frequency of carriage of any NOD2 allelic variant was 35% in Crohn's disease patients compared with 11% in ulcerative colitis patients (p=0.001). Within the combined CD cohort, the frequency of homozygotes with the 3020insC mutation and of compound heterozygotes was 1% and 4%, respectively, while none of the ulcerative colitis patients had such a genotype. We conclude from these data that allelic variants of NOD2 are associated with Crohn's disease across diverse geographic and ethnically-defined patient populations.

Table 1. Clinical characteristics of Crohn's disease cohorts.

Clinical characteristics	CD1	CD2	
	n=142	n=59	p
Gender (M/F)	79/63	33/26	0.969
Age at onset	22 (4-67)	22 (2-58)	0.6621
Ethnicity (Jew/Non-Jew)	60/82	23/36	0.668
Disease location (%)			
SB only	19.0	26.4	0.496
Colon only	20.4	20.8	
SB and Colon	60.6	52.8	
Perianal perforating (%)	35.9	28.8	0.332

Internal perforating (%)	47.2	23.7	0.002
Fibrostenosing disease (%)	59.9	30.5	0.001
UC-like (%)	39.4	22.0	0.018
pANCA-positive (%)	19.7	12.5	0.295
ASCA-positive* (%)	57.0	38.6	0.019

* Two patients in the second cohort did not undergo ASCA testing.

Table 2. Frequency of NOD2 allelic variants in two cohorts of patients with Crohn's disease (CD1 and CD2) and ulcerative colitis.

Allelic variants	**UC (n=175)**	**CD1 (n=142)**	**CD2 (n=59)**	**Combined CD (n=201)**	**p (UC vs. Combined CD)**
R675W (SNP 8)	5.7%	16.9%	15.3%	16.4%	0.001
G881R (SNP 12)	1.7%	12.0%	10.2%	11.4%	0.0001
3020insC (SNP 13)	3.4%	11.3%	11.9%	11.4%	0.004
Carriage of any allelic variant	10.9%	36.6%	32.2%	35.3%	0.001

Mutations of NOD2 are associated with fibrostenosing Crohn's disease

Patients with Crohn's disease express diverse clinical phenotypes that may be due to differences in underlying genetic factors. We hypothesized that

mutations in NOD2 may be associated with specific Crohn's disease-related clinical phenotypes. We further hypothesized that mutations in NOD2 may be associated with specific Crohn's disease-related serum immune markers. To test these hypotheses, we performed univariate analyses evaluating the association between NOD2 allelic variants and pre-defined clinical characteristics including age of onset, disease location and disease phenotype (i.e. fibrostenosing disease, internal-perforating disease, perianal fistulizing disease or UC-like disease). Additionally, we tested the association between NOD2 allelic variants and expression of the serum immune markers ASCA and pANCA. Univariate analysis revealed that the CD-related NOD2 variants were significantly associated with fibrostenosing disease in Cohort 1 ($p=0.049$) for the three allelic variants combined (Table 3). A positive association at a less stringent significance level ($p< 0.1$) was also observed with small bowel involvement and younger age of onset and a negative association with UC-like disease in this cohort. With respect to serologic markers, patients with the insertion mutation 3020insC were more likely to express ASCA ($p=0.053$).

Table 3. Relationship of NOD2 allelic variants and clinical phenotypes of Crohn's disease in cohort 1 (CD1).

Clinical phenotypes		n	Qualitative Trait %NOD2 variant carriers			
			R675W (SNP 8)	G881R (SNP 12)	3020insC (SNP 13)	Carriage of any allelic variant
Small bowel involvement	yes	113	19.5%	11.5%	14.2%	40.7%
	no	29	6.9%	13.8%	0.00%	20.7%
p			0.081	0.494	**0.04**	**0.063**
Perianal perforating	yes	51	11.8%	11.8%	13.7%	35.3%
	no	91	19.8%	12.1%	9.9%	37.4%
p			0.248	0.839	0.547	0.747
Internal perforating	yes	67	13.4%	16.4%	11.9%	37.3%
	no	75	20.0%	8.0%	10.7%	36.0%
p			0.346	0.178	0.91	0.96
Fibrostenosing	yes	85	18.8%	14.1%	15.3%	43.5%
	no	57	14.0%	8.8%	5.3%	26.3%
p			0.389	0.458	**0.084**	**0.049**
UC-like	yes	56	17.9%	10.7%	5.4%	30.4%
	no	86	16.3%	12.8%	15.1%	40.7%
p			0.822	0.736	**0.076**	0.22
pANCA positive	yes	28	17.9%	14.3%	7.1%	32.1%
	no	114	16.7%	11.4%	12.3%	37.7%

p		0.82	0.793	0.394	0.529
ASCA positive	yes 81	18.5%	9.9%	16.1%	38.3%
	no 61	14.8%	14.8%	4.9%	34.4%
p		0.467	0.234	**0.053**	0.744

		Quantitative Trait median (range)			
		R675W (SNP 8)	G881R (SNP 12)	3020ins C (SNP 13)	Carriage of any allelic variant
Age of onset					
carrier of NOD2 variant	yes	22 (6-67)	22 (4-62)	19 (10-50)	20 (4-67)
	no	22 (4-63)	22 (4-67)	22 (4-67)	22 (4-63)
p		0.715	0.937	**0.074**	0.238

Based on our data in the first cohort, we hypothesized that NOD2 variants were positively associated with fibrostenosing Crohn's disease, small bowel involvement, ASCA positivity, and younger age of onset and negatively associated with UC-like disease. In cohort 2, we tested these specific hypotheses generated from cohort 1. As with cohort 1, cohort 2 demonstrated a significant association between NOD2 allelic variants and fibrostenosing disease (p=0.002, with Bonferroni correction p=0.01) (Table 4). When the two cohorts were analyzed together, the association between NOD2 variants and fibrostenosing disease was even more significant (p= 0.001) (Figure 1). These relationships were observed in

both Jews and non-Jews. Approximately 46% of CD patients with fibrostenosing disease (Jews 52% vs. non-Jews 42%) have at least one of these rare alleles as compared to only 23% (Jews 21.6% vs. non-Jews 25%) of CD patients without fibrostenosing disease (OR 2.8, 95% CI 1.56-5.18) (Figure 1). Of the three rare alleles, the frameshift mutation, 3020insC, demonstrated the greatest association with fibrostenosing disease (47% vs. 17%, p for cohorts combined= 0.006).

Table 4. Relationship of NOD2 allelic variants and clinical phenotypes of Crohn's disease in cohort 2 (CD2).

Clinical phenotypes			Qualitative Trait %NOD2 variant carriers			
		n	R675W (SNP 8)	G881R (SNP 12)	3020insC (SNP 13)	Carriage of any allelic variant
Fibrostenosing	yes	18	22.2%	22.2%	27.8%	61.1%
	no	41	12.2%	4.9%	4.9%	19.5%
p			0.315	**0.048**	**0.018**	**0.002**
Small bowel involvement	yes	42	19.1%	9.5%	14.3%	35.7%
	no	17	5.9%	11.8%	5.9%	23.5%
p			0.22	0.828	0.288	0.354
UC-like	yes	13	7.7%	15.4%	7.7%	23.1%
	no	46	17.4%	8.7%	13.0%	34.8%
p			0.399	0.489	0.593	0.432

ASCA positive	yes	22	9.1%	13.6%	13.6%	31.8%
	no	35	17.1%	8.6%	11.4%	31.4%
p			0.4	0.542	0.735	0.956

			Quantitative Trait median (range)			
			R675W (SNP 8)	G881R (SNP 12)	3020insC (SNP 13)	Carriage of any allelic variant
Age of onset						
carrier of NOD2 variant	yes		27 (10-58)	26 (7-33)	17 (13-35)	22 (7-58)
	no		19 (2-55)	20 (2-58)	24 (2-58)	22 (2-55)
p			0.332	0.9	0.566	0.981

<u>Figure 1. Rare allelic variants of NOD2 are associated with fibrostenosing Crohn's disease</u>. Two cohorts of Crohn's disease patients were genotyped for three allelic variants of NOD2. Carriage of these allelic variants was more frequent in Crohn's disease patients with fibrostenosing disease compared with those that did not have fibrostenosing disease.

We next analyzed the risk of fibrostenosing disease in Crohn's disease patients carrying homozygous mutations or compound heterozygous mutations in NOD2. Compared with patients who were not carriers of NOD2

mutations, patients who were carriers of two mutations in NOD2 were significantly more likely to demonstrate fibrostenosing disease (85% vs. 43%, OR 7.4, 95%CI 1.9-28.9, p=0.004) (Figure 2). Patients who were carriers of a single NOD2 mutation were also significantly more likely to demonstrate fibrostenosing disease when compared with patients who were not carriers of NOD2 mutations (64% vs. 43%, OR 2.37, 95%CI 1.26-4.47, p= 0.008). The patients with fibrostenosing disease in these two cohorts could be characterized as having only fibrostenosing disease, or both fibrostenosing and perforating disease since these two phenotypes often occur in the same patient. The percent of NOD2 variants in patients with fibrostenosing disease only was 48.3% which was similar to that seen in patients with both fibrostenosing and perforating complications (46.0%, p=0.8). When we compared patients with fibrostenosing disease to those patients described as having perforating disease only (perianal or internal), the percent carriage of NOD2 allelic variants in patients with fibrostenosing disease (with or without perforating complications) (46.6%) was much greater than that seen in patients with only perforating complications (18.6%, p=0.002) (Figure 3).

Figure 2. Frequency of fibrostenosing complications in patients with NOD2 allelic variants. **Based on genotyping for the three rare alleles of NOD2, patients could be described as carrying 0, 1 or 2 rare alleles (x-axis). Left Y axis demonstrates the frequency of fibrostenosing complications (●); *p=0.008, **p=0.004 compared with 0**

alleles. Right Y axis demonstrates the odds ratio (◆) with 95% confidence intervals in parentheses.

Figure 3. Comparison of NOD2 allelic frequencies in patients with fibrostenosing disease compared with perforating disease. Patients were separated by the presence of fibrostenosing disease with (Fib+perf) or without (Fib only) perforating complications and compared with patients with perforating complications and without evidence of fibrostenosis (Perf only). Patients with evidence of fibrostenosis were significantly more likely than those with purely perforating disease to carry NOD2 allelic variants in each of the cohorts and in the combined cohorts.

Studies performed on large European cohorts of Crohn's disease patients have demonstrated an association between carriage of NOD2 allelic variants and small bowel involvement [30, 40], a negative association with colonic involvement [24] as well as younger age of onset [24, 30]. In our cohorts, a trend towards small bowel involvement was seen in the first cohort (40.7% vs. 20.7%) (Table 3) and again in the second cohort (35.7% vs. 23.5%) (Table 4). When the two cohorts were analyzed together, small bowel involvement was found to be significantly associated with carriage of NOD2 variants (39.4% versus 21.7%, p=0.036). As expected, a negative trend was seen between carriage of NOD2 variants and UC-like Crohn's disease for the cohorts combined (OR 0.37, 95% CI 0.12-1.09, p=0.071). The second cohort did not, however, demonstrate an association between NOD2 variants and ASCA positivity. In

addition, while the younger age of onset appeared to be associated with the 3020insC allele of NOD2 in the first cohort (p=0.074), this association was not significant in the second cohort. In the combined CD cohorts, this association demonstrated a borderline significance (p=0.062). In multivariate analysis, all variables with at least borderline signficance (<0.1) in either cohort were tested simultaneously for their association with NOD2 allelic variants using logistic regression. As shown in Table 5, the only phenotype that was significantly associated with NOD2 (p<0.05) was fibrostenosing disease (OR=2.8, 95%CI: 1.3~6.0). In summary, these data demonstrate that fibrostenosing disease is independently associated with NOD2 allelic variants regardless of ethnic background and other clinical phenotypes.

Because fibrostenosing disease is more likely to occur in patients with small bowel involvement, we stratified patients based on small bowel involvement to further address the primary association between fibrostenosing disease and NOD2 variants. Among patients with small bowel involvement, 26.4% of patients who do not have fibrostenosing disease (n=53) have a NOD2 variant while a much greater percentage (46.1%) of patients who had fibrostenosing disease (n=102) have a NOD2 variant (p=0.017). A similar trend was observed among patients without small bowel involvement (p=0.05) and the combined analysis conditioning on small bowel involvement yielded a significance level of 0.009. However, after controlling for fibrostenosing disease, small bowel involvement was not associated with NOD2

variants (p=0.63). This result agrees with the results from the logistic regression analysis and suggests that the association between fibrostenosing disease and NOD2 variants is independent of small bowel involvement, but the observed small bowel association with NOD2 is secondary to the presence of fibrostenosing disease.

Table 5. Multivariate analysis in the combined cohort for five phenotypic variables. Multivariate analysis was performed for the five phenotypes that demonstrated an association by univariate analyses. Of the variables tested, only fibrostenosing disease demonstrated an independent association with NOD2 allelic variants.

Clinical phenotypes	OR	95% CI	p
Fibrostenosing disease	2.8	1.3-6.0	0.011
Small bowel involvement	1.3	0.5-3.4	0.561
UC-like	0.9	0.4-1.7	0.658
ASCA positive	0.7	0.3-1.3	0.250
Age of onset	1.0	0.9-1.0	0.874

Discussion

Crohn's disease is a multigenic disorder with diverse clinical manifestations. Several population-based studies have described an association of NOD2 gene mutations in Crohn's disease [21-23]. This study describes a genotype-phenotype association for NOD2 allelic variants in Crohn's disease. Specifically, we describe an association between the presence of NOD2 mutations and small bowel stricturing Crohn's disease. Both the genetic association and its phenotypic association with

fibrostenosis was observed in Jews and non-Jews with similar frequency. The finding of a Crohn's disease subtype in patients carrying NOD2 mutations provides biological evidence for the NOD2 gene in the pathogenesis of Crohn's disease and lends further support for NOD2 as a Crohn's disease susceptibility gene. Alternatively, these findings may be due to the effect of a neighboring gene in linkage disequilibrium with NOD2. The NOD2 gene is also the first that has been described to be associated with fibrostenosing Crohn's disease. Basic and translational studies will need to explore whether CD-associated NOD2 mutations directly alter the path to fibrogenesis in response to small bowel inflammation.

In designing this study, we explored a variety of previously described clinical phenotypes within Crohn's disease [2, 3] and their association with NOD2 allelic variants. A major strength of our study lies in the ability to analyze two independent cohorts of Crohn's disease patients demonstrating similar carriage of NOD2 allelic variants. By setting a less stringent significance level in the first cohort, we were able to increase our power to detect clinical associations with NOD2 mutations. The second cohort was then used to confirm the associations found in the first cohort and reduce type 1 error. The consistency of the NOD2 genetic and phenotypic association in our cohorts as well as the European cohorts [24, 30] speaks strongly to the specific role of NOD2 mutations in the development of fibrostenosing complications. Recently, other groups have described that NOD2 mutations are associated primarily with ileal disease [24, 30, 40]. In our conditioning analysis,

carriage of NOD2 mutations was no more likely in patients with small bowel involvement without stricturing complications (26.4%) than in Crohn's disease overall. By contrast, patients with small bowel involvement and stricturing complications were significantly more likely to have carriage of NOD2 allelic variants (46.1%, p=0.017). We, therefore, believe that mutations in NOD2 are not just associated with small bowel involvement but rather with the subset of patients whose small bowel disease becomes fibrostenotic. Other groups have also identified younger age of onset in patients with NOD2 mutations [24, 30]. Although we did not find a similar association, it is likely that our study did not have the power to detect this smaller association.

In addition to demonstrating an association between NOD2 mutations and fibrostenosis, our data demonstrate that NOD2 mutations are not associated with perforating complications of Crohn's disease. Indeed, even in patients who can be described as having both fibrostenotic complications, e.g. small bowel obstruction, and perforating complications, e.g. perianal fistula, these patients are genetically similar to those with fibrostenosis only (Figure 3). These data suggest that NOD2 contributes to the pathogenesis of fibrostenosis regardless of other superimposed complications such as perforations. Little is known about the genetic or immunologic factors resulting in fibrostenosis in Crohn's disease. Compared with Crohn's patients requiring surgery for perforating complications of disease, patients with fibrostenotic disease have a longer interval between surgeries [31, 41, 42]. Studies have

demonstrated that fibroblasts isolated from strictures of patients with Crohn's disease produce significantly more type III collagen than fibroblasts isolated from non-strictured Crohn's disease lamina propria [43, 44]. Collagen production by intestinal fibroblasts is regulated by transforming growth factor beta 1 (TGF-beta 1), TGF-beta-2, platelet derived growth factor and IL-1 [45, 46]. We have previously described that high levels of ASCA and expression of both IgG and IgA sub-types are highly associated with fibrostenosing disease and multiple surgeries [2]. ASCA is itself a heritable trait and is expressed by unaffected family members of ASCA-positive Crohn's disease patients [4, 47]. Linkage studies have demonstrated that ASCA levels are linked with the major histocompatibility complex on chromosome 6, but not with the IBD1 locus (NOD2 gene region) on chromosome 16 [48]. Because fibrostenosing disease occurs more commonly in the small bowel and is associated with the serum immune marker ASCA [2], we examined the association of NOD2 mutations with ASCA positivity. We did not identify a significant association between NOD2 variants and expression of ASCA. These data are consistent with our previous negative linkage results with the IBD1 locus [48] and suggest that the factors downstream of NOD2 that result in fibrostenosis are distinct from those resulting in ASCA positivity.

In vitro studies have demonstrated that expression of the truncated mutant of NOD2 (3020insC or 980fs) results in diminished LPS responsiveness [22]. The functional phenotype of the two missense mutations in NOD2, R675W and G881R, and the additional 27 rare

mutations with respect to LPS responsiveness is not clear but may result in other defects in innate immunity [24]. Although all three allelic variants were independently associated with Crohn's disease (Table 2), carriage of the truncation mutation of NOD2 was most strongly correlated with fibrostenosing disease. One model to reconcile diminished LPS responsiveness with NOD2 mutations and the pathogenesis of Crohn's disease is a defective response to pathogenic or commensal organisms resulting in a chronic infection or an aberrant immune response. Since NOD2 is an intracellular protein primarily expressed by monocytes, mutations in NOD2 may predispose the susceptible host to a chronic infection with an intracellular pathogen [49, 50]. Given the diversity of microbes in the gut, it is also possible that NOD2 variants may increase pro-inflammatory responses to specific bacteria or bacterial products other than LPS. Because we have described an association between fibrostenosing disease and NOD2 variants, we hypothesize that the immune response distal to a mutation in NOD2 shifts T cells towards TGF-□ cytokine production and increases collagen deposition by smooth muscle cells and fibroblasts in the intestine.

Footnote:

Stephan R. Targan, M.D. is a founder and has an equity interest in Prometheus Laboratories.

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What is claims is:

1. A method of diagnosing or predicting susceptibility to a clinical subtype of Crohn's disease (CD) characterized by fibrostenosing disease, comprising:

determining the presence or absence in a patient with CD of a fibrostenosis-associated allele linked to an NOD2 locus,

wherein the presence of said fibrostenosis-associated allele indicates said clinical subtype of CD characterized by fibrostenosing disease.

2. The method of claim 1, wherein said fibrostenosis-associated allele is associated with Crohn's disease.

3. The method of claim 1, wherein said fibrostenosis-associated allele is within said NOD2 locus.

4. The method of claim 3, wherein said fibrostenosis-associated allele is within the NOD2 coding sequence.

5. The method of claim 4, wherein said fibrostenosis-associated allele is selected from the group consisting of R675W (SNP8), G881R (SNP12) and 3020insC (SNP13).

6. The method of claim 1, comprising determining the presence or absence in a patient with CD of at least two fibrostenosis-associated alleles linked to an NOD2 locus,

wherein the presence of one or more of said fibrostenosis-associated alleles indicates a clinical subtype of CD characterized by fibrostenosing disease.

7. The method of claim 6, wherein said at least two fibrostenosis-associated alleles are selected from the group consisting of R675W (SNP8), G881R (SNP12) and 3020insC (SNP13).

8. The method of claim 1, wherein determining the presence or absence of said fibrostenosis-associated allele comprises enzymatic amplification of nucleic acid from said patient with CD.

9. The method of claim 8, wherein said nucleic acid is amplified with a primer set selected from the group consisting of:

5'-GGC GGG ATG GAG TGG AA-3' / 5'-CTG GCT GAG TGC CAG ACA TCT-3';

5'-CCA CCT CAA GCT CTG GTG ATC-3'/ 5'-GTT GAC TCT TTT GGC CTT TTC AG-3; and

5'-CCT TAC CAG ACT TCC AGG ATG GT-3' / 5'-TGT CCA ATA ACT GCA TCA CCT ACC T-3'.

10. The method of claim 1 or claim 8, wherein determining the presence or absence of said fibrostenosis-associated allele comprises allele-specific hybridization, allele-specific nucleotide incorporation, allele-specific oligonucleotide ligation or allele-specific invasive cleavage.

11. The method of claim 5, wherein determining the presence or absence of said fibrostenosis-associated allele comprises hybridization with

5'FAM-TGC TCC GGC GCC A-MGBNFQ and
5'TET-CTG CTC TGG CGC CA-MGBNFQ.

12. The method of claim 5, wherein determining the presence or absence of said fibrostenosis-associated allele comprises hybridization with

5'FAM-CTG TGT TGC CCC AGA A-MGBNFQ and
5'TET-CTC TGT TGC GCC AGA-MGBNFQ.

13. The method of claim 5, wherein determining the presence or absence of said fibrostenosis-associated allele comprises hybridization with

5'FAM-CCT TTC AAG GGG CCT-MGBNFQ and
5'TET-CTT TCA AGG GCC TGC-MGBNFQ.

14. The method of claim 1, further comprising determining the presence or absence of ASCA in said patient with CD.

15. The method of claim 1, wherein said clinical subtype of Crohn's disease is characterized by fibrostenosing disease independent of small bowel involvement.

16. A method of preventing or reducing the severity of fibrostenosing disease in a patient with CD, comprising enhancing the expression or activity of NOD2 in said patient with CD.

17. The method of claim 16, comprising administering to said patient with CD a nucleic acid molecule encoding NOD2 or an active fragment thereof.

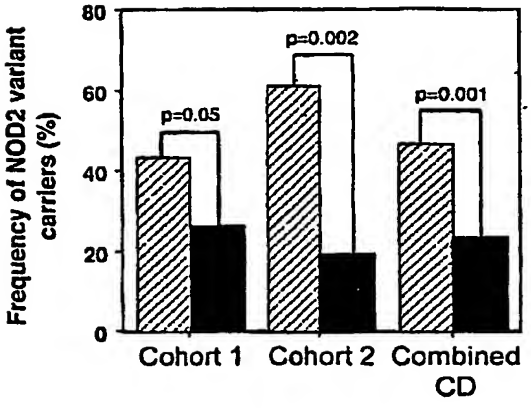
Frequency of NOD2 variant carriers (%)
80
60
40
20
0
p=0.05
p=0.002
p=0.001
Cohort 1
Cohort 2
Combined CD

FIGURE 1

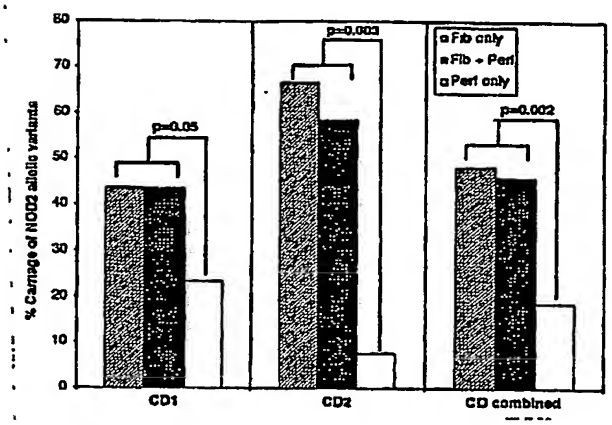
% Carriage of NOD2 allelic variants
80
70
60
50
40
30
20
10
0
p=0.05
p=0.003
p=0.002
Fib only
Fib + Perf
Perf only
CD1
CD2
CD combined

FIGURE 2

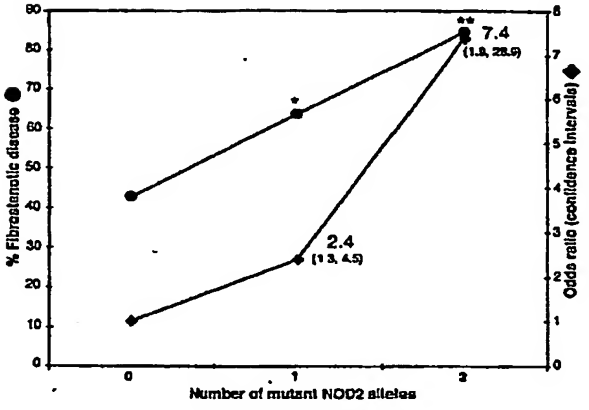
% Fibrostenotic disease
Odds ratio (confidence intervals)
Number of mutant NOD2 alleles
7.4
(1.9, 28.5)
2.4
(1.3, 4.5)
*
**
0
1
2
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6
7
8

FIGURE 3